# EXHIBT 1

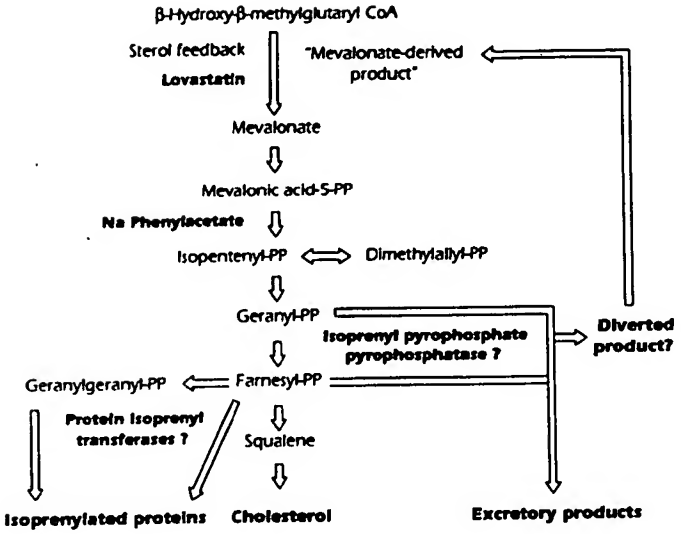


***FIGURE 1*** An abbreviated outline of the mevalonate pathway. Lovastatin, sodium phenylacetate and, potentially, protein isoprenyl transferase inhibitors, targeted to tumor cells offer novel approaches to cancer chemotherapy. Studies reviewed in the text suggest that end products of plant mevalonate pathways modulate mevalonate synthesis via regulatory actions similar to those attributed to the mevalonate-derived product by Goldstein and Brown (1990).

## *Dietary isoprenoids and cardiovascular disease risk*

Our anomalous finding that fiber-rich grains, contrary to the action of bile acid binding agents, suppress cholesterol synthesis led to the determination that a minor constituent of barley, α-tocotrienol, elicits a dose-dependent suppression of HMGR activity with concomitant decreases in serum cholesterol (reviewed in Elson and Yu 1994a). Other mevalonate-derived end products of plant secondary metabolism (isoprenoids,[4] (**Fig. 2**) similarly suppress HMGR activity and lower serum cholesterol (Fitch et al. 1989, Qureshi et al. 1988, Yu et al. 1994). Unlike the competitive inhibitors of HMGR activity (Goldstein and Brown 1990), the inhibitory action of the tocotrienols (Parker et al. 1993, Pearce et al. 1992), farnesyl acetate (Bradfute and Simoni 1994), β-carotene (Moreno et al. 1994) and the cyclic monoterpenes (Clegg et al. 1982) is mediated via a decrease in enzyme mass. A posttranscriptional action is postulated because the tocotrienols, β-carotene and farnesyl acetate do not lower HMGR mRNA levels, whereas both reductase mass and activity are substantially decreased. Thus, the regulatory actions introduced by these isoprenoids reflect those of the mevalonate-derived product. The diversity of the HMGR-suppressive isoprenoids studied in our lab and their ties to a common precursor, mevalonic acid, are shown in Figures 2 and 3. Farnesol and geraniol are structurally related to the side chain of the tocotrienols; geranyl pyrophosphate and farnesyl pyrophosphate, respectively, are intermediates in the synthesis of monoterpenes and sesquiterpenes. The placement

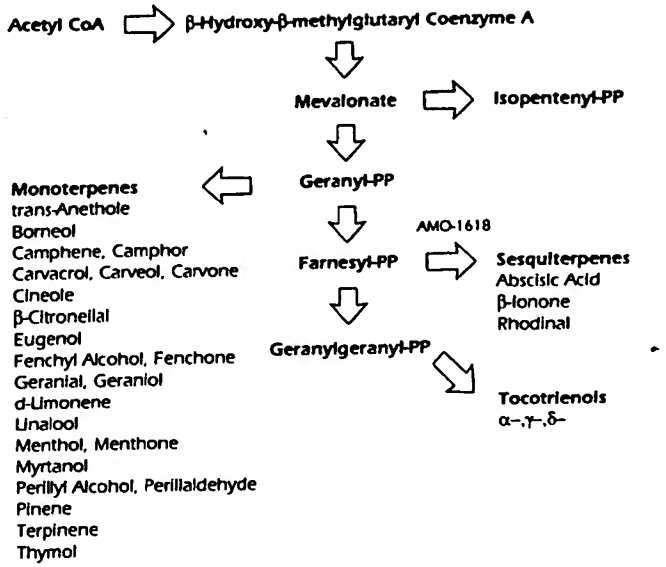


***FIGURE 2*** An overview of the common roots of mevalonate-suppressive isoprenoids; farnesyl acetate (Bradfute and Simoni 1994) and β-carotene (Moreno et al. 1994) similarly suppress mevalonate synthesis.

of the double bond and head-to-head or head-to-tail coupling of the 5-carbon intermediates determines the secondary structural characteristics of the monoterpenes (**Fig. 3**). Cholesterol-lowering actions of more complex isoprenoids and of plant materials likely to be rich sources of similar isoprenoids are widely reported in the literature.

## *Cancer: mevalonate pathway pharmacology*

Competitive inhibitors of HMGR activity arrest cells at the G1/S interface of the cell cycle. This block

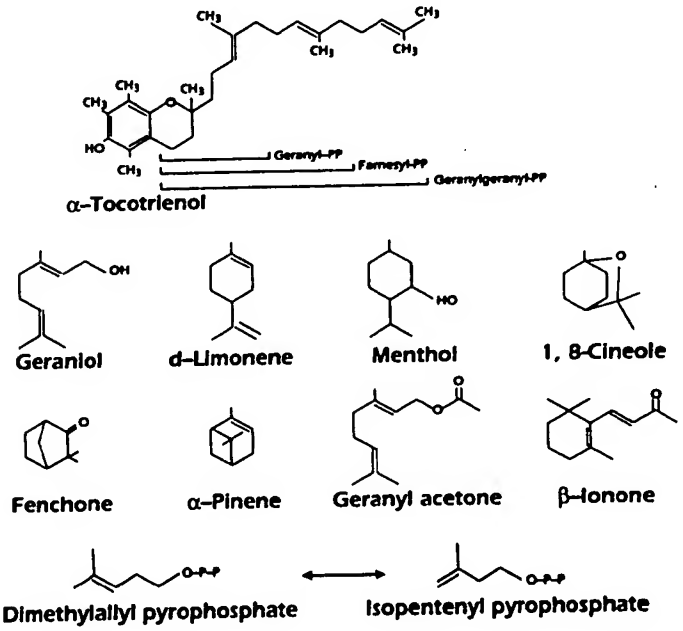


***FIGURE 3*** The structural diversity of mevalonate-suppressive isoprenoids. Fragments of the tocotrienol molecule can be viewed as precursors of the monoterpenes, sesquiterpenes and carotenoids. Head-to-head and head-to-tail unions of the five-carbon isomers and specific oxidases determine the ultimate structures of the isoprenoids.

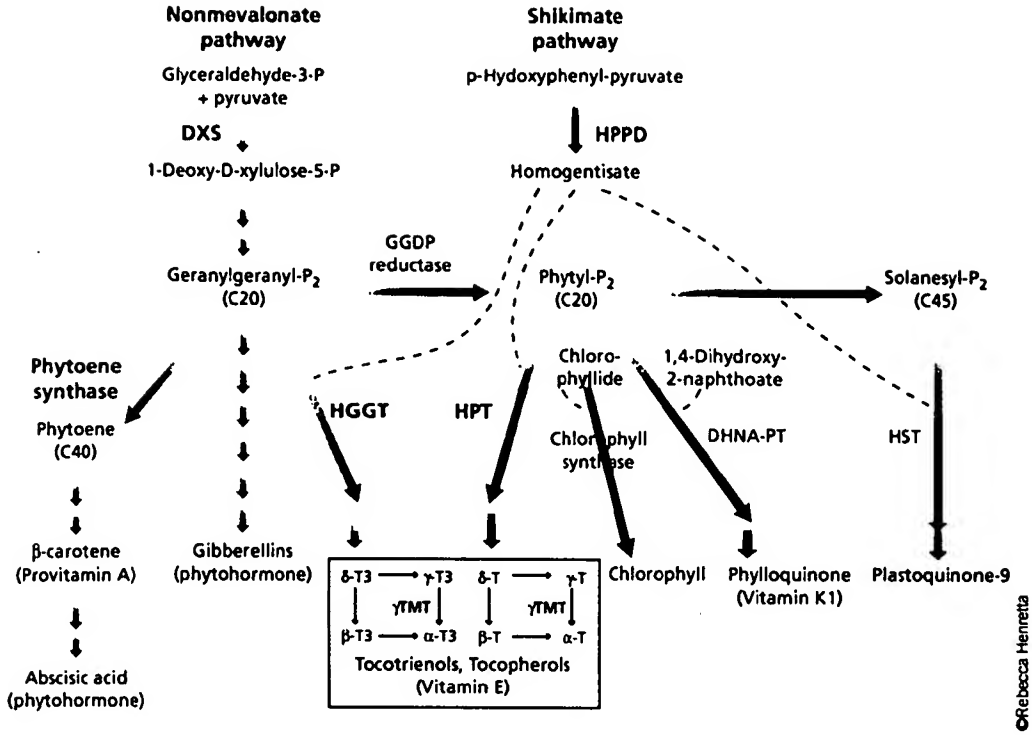


**Figure 1** Synthesis of prenyl lipids in plants. Activated isoprenoids (phytyl diphosphate, geranylgeranyl diphosphate, solanesyldiphosphate) derived from the plastid nonmevalonate pathway are the precursors for the synthesis of prenyl lipids in plants, some of which have an important function for human nutrition (vitamin E, vitamin K1, provitamin A). Enzymatic steps that have been manipulated in transgenic plants to boost prenyl lipid synthesis are depicted in bold. The HGGT pathway described by Cahoon *et al.*[2] is shown in red. Abbreviations: DHNA-PT, 1,4-dihydroxy-2-naphthoate phytyltransferase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGDP reductase, geranylgeranyl diphosphate reductase; HGGT, homogentisic acid geranylgeranyltransferase; HPPD, *p*-hydroxyphenyl-pyruvate dioxygenase; HPT, homogentisic acid phytyltransferase; HST, homogentisic acid solanesyltransferase; P, phosphate; T, tocopherol; T3, tocotrienol; γ-TMT, γ-tocopherol methyltransferase.

observed for tocopherol synthesis in plants overexpressing HPT[2,6].

HGGT belongs to a family of plant prenyltransferases that includes HPT and chlorophyll synthase[2]. Tocotrienols are highly abundant in seeds of monocot plants, such as cereals, but mostly absent from dicots. Furthermore, HGGT sequences from rice, barley and wheat are more closely related to HPT sequences from monocots than from dicots[2], suggesting that the monocot prenyltransferases might have a common evolutionary ancestor. Interestingly, the plant HPT enzymes have been shown to be specific for phytyl diphosphate, but to discriminate against geranylgeranyl diphosphate, whereas the cyanobacterial HPT enzyme is active with both substrates[3]. On the basis of data obtained in transgenic plants, it is likely that HGGT is specific for geranylgeranyl diphosphate[2]. However, direct evidence for this awaits characterization of the *in vitro* substrate specificity of HGGT.

The 'activity of vitamin E' historically has been defined by the rat fetal reabsorption assay. Because of different affinities to the α-tocopherol transport protein in the blood plasma, which determine the bio-availability of vitamin E molecules, the forms of tocopherols and tocotrienols significantly differ in vitamin E activity, with α-tocopherol showing the highest activity set to 'one α-tocopherol equivalent' (1 α-TE)[1]. The α-TE values for γ-tocopherol, α-tocotrienol and γ-tocotrienol are 0.1, 0.3 and 'below detection,' respectively. Because overexpression of HGGT in plants mostly results in an increase in γ-tocotrienol with the other vitamin E forms remaining constant[2], an elevation in the total amount of tocopherols/tocotrienols by a factor of 10 translates into a much smaller increase in vitamin E activity, as measured in α-TE units. However, the relevance of the traditional rat fetal reabsorption assay for the diverse biological functions of tocopherols and tocotrienols in human nutrition is questionable. Furthermore, *in vitro* studies have demonstrated that tocotrienols exert a much stronger antioxidant activity[10], which might be the reason for their superior therapeutic effects in certain clinical contexts, such as hypercholesterolemia, thrombosis and cancer[11].

Not surprisingly, an entire industry has developed around the production and commercialization of tocotrienols as 'nutraceuticals.' Because of their high antioxidant activity, an increase in tocotrienols is expected to extend the shelf life of vegetable oils used for human nutrition. Furthermore, transgenic plants accumulating tocotrienols in their leaves might show a higher resistance to oxidative stresses originating from drought and heat. The conversion of a large fraction of γ-tocotrienol accumulating in HGGT transgenic plants into α-tocotrienol (which has a higher vitamin E activity; see above) might be achieved via introduction of a second transgene, γ-tocopherol methyltransferase (γ-TMT). γ-TMT has been shown to be critical for the last methylation step in the production of α-tocopherol and α-tocotrienol[12].

Genetic engineering of crop plants for the production of high levels of tocotrienols raises the issue of whether carbon flux into other important nutraceuticals produced via the plastid prenyl lipid pathway (*e.g.*, provitamin A and vitamin K) might be affected. It has already been shown that an increase in carotenoids by overexpression of phytoene synthase results in a reduction of tocopherol and chlorophyll in seeds of rape[8]. Finally, the health benefits proposed for the individual forms of tocotrienols and tocopherols are still under debate, and further research is needed to assign specific roles to each vitamin E form in human nutrition.


1. Food and Nutrition Board, Institute of Medicine. in *Dietary Intakes for Vitamin C, Vitamin E, Selenium and Carotenoids*. 186–283 (National Academy Press, Washington, DC, 2000).
2. Cahoon, E.B. *et al. Nat. Biotechnol.* **21**, 1082–1087 (2003).
3. Collakova, E. & DellaPenna, D. *Plant Physiol.* **127**, 1113–1127 (2001).
4. Porfirova, S. *et al. Proc. Natl. Acad. Sci USA* **99**, 12495–12500 (2002).
5. Falk *et al. FEBS Lett.* **540**, 35–40 (2003).
6. Collakova, E. & Della Penna, D. *Plant Physiol.* **131**, 632–642 (2003).
7. Estévez J.M. *et al. J. Biol. Chem.* **276**, 22901–22909 (2001).
8. Shewmaker *et al. Plant J.* **20**, 401–412 (1999).
9. Ye, X. *et al. Science* **287**, 303–305 (2000).
10. Fryer, M.J. *Plant Cell Environ.* **15**, 381–392 (1992).
11. Theriault, A. *et al. Clinic. Biochem.* **32**, 309–319 (1999).
12. Shintani, D. & DellaPenna, D. *Science* **282**, 2098–2100 (1998).

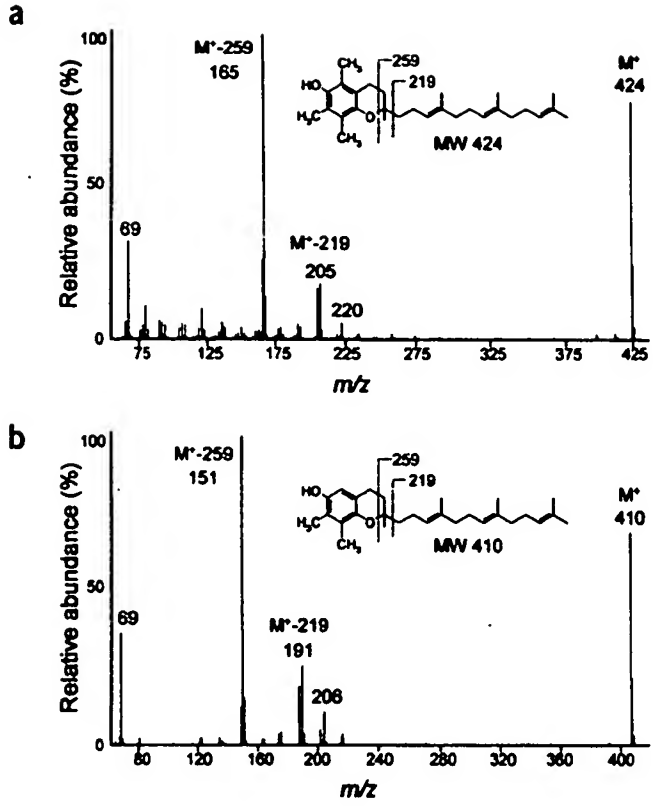


Figure 5 Mass spectra of α-tocotrienol and γ-tocotrienol from organic extracts of tobacco callus expressing the barley HGGT cDNA. (a) α-tocotrienol. (b) γ-tocotrienol. The mass spectra contain molecular ions for α-tocotrienol (*m/z* 424) and γ-tocotrienol (*m/z* 410) as well as M+-219 ions arising from loss of the side chain and M+-259 ions arising from cleavage of the chromanol ring and accompanying rearrangement as described[35].

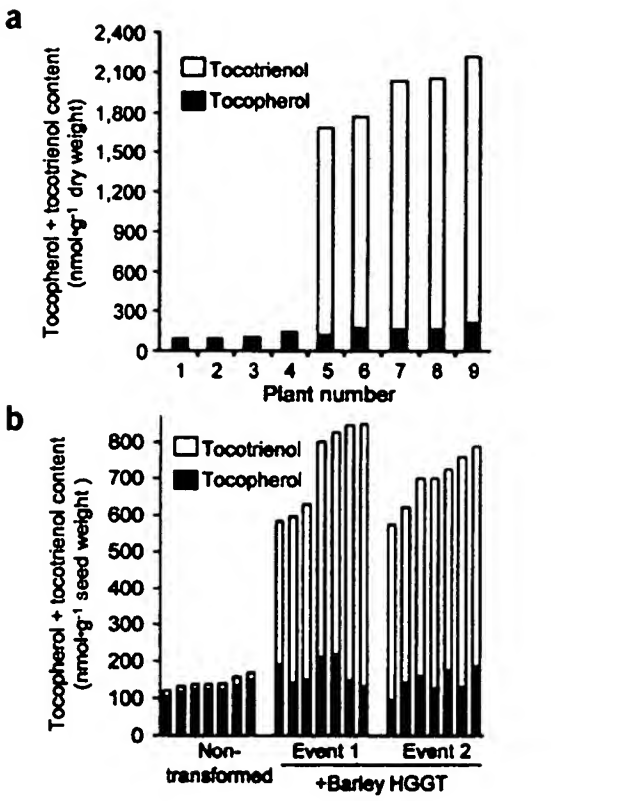


Figure 6 Tocopherol and tocotrienol content of *Arabidopsis thaliana* leaves and corn seeds expressing the barley HGGT cDNA. (a) Tocopherol and tocotrienol content of lyophilized *A. thaliana* leaves from nine $T_2$ plants from a selected line that was transformed with the barley HGGT cDNA linked to the CaMV 35S promoter. The analyzed plants included wild-type segregants that did not accumulate tocotrienols, as indicated in the graph (i.e., Plants 1, 2, 3, 4). (b) Tocopherol and tocotrienol content of single corn seeds from a nontransformed plant and two transgenic events. The transgenic plants were the initial $R_0$ generation, and the measurements shown are from seven single trait–positive seeds.

shown to confer tocotrienol biosynthetic ability to a variety of plant tissues that do not normally produce this form of vitamin E. By contrast, HPTs from plant tissues that produce only tocopherols have no demonstrated *in vitro* or *in vivo* ability to divert metabolic flux into the synthesis of tocotrienols[17–20,22]. Our findings therefore not only demonstrate a method for producing tocotrienols in transgenic plants, but also provide the first evidence for the synthesis of tocotrienols in plants through the HGGT-catalyzed pathway shown in Figure 1.

The increases in total content of tocotrienols and tocopherols achieved in this study are markedly greater than those obtained earlier by other transgenic approaches. Most notably, overexpression of the *A. thaliana* HPT resulted in a 4.4-fold and two-fold increase in the tocopherol content of *A. thaliana* leaves[23] and seeds[20], respectively. In our study, a 10- to 15-fold increase in the tocotrienol and tocopherol content of *A. thaliana* leaves was obtained by overexpression of the barley HGGT. Similarly, embryo-specific overexpression of the barley HGGT cDNA in corn seeds yielded up to a six-fold increase in tocotrienol and tocopherol content. This elevation of tocotrienol and tocopherol content resulted almost entirely from the production of tocotrienols rather than from increases in amounts of tocopherol. This *in vivo* activity is consistent with a strong substrate preference for GGDP rather than PDP, as predicted for an HGGT enzyme (Fig. 1). We attempted to confirm this apparent substrate specificity by direct assay of recombinant barley HGGT. However, we were unable to generate detectable levels of this membrane-associated enzyme in *Escherichia coli* cells using a T7 RNA polymerase promoter to drive expression and were unable to measure activity from extracts of these cells using $^3$H-labeled HGA and GGDP or PDP as substrates.

Our results also show conclusively that the introduction of only one metabolic step is sufficient to engineer tocotrienol biosynthetic ability in transgenic plant cells. This finding confirms earlier reports that the 2-methyl-6-geranylgeranylbenzoquinol product of HGGT (Fig. 1) can be metabolized *in vitro* by methyltransferase and cyclase enzymes from the tocopherol biosynthetic pathway to form tocotrienols[17,18,22,27]. Of note, γ-tocotrienol rather than the more methylated α-tocotrienol was the major form of tocotrienol in transgenic plant tissues that accumulated the highest levels of these antioxidants. This form of tocotrienol has low dietary vitamin E activity relative to α-tocopherol and α-tocotrienol as shown by the rat gestation-resorption assay[6], the classic measurement of vitamin E activity. The high levels of γ-tocotrienol in the transgenic tissues likely indicate that HGGT-catalyzed flux into tocotrienol synthesis exceeded the catalytic capacity of γ-tocopherol methyltransferase, the enzyme that converts γ-tocopherol into α-tocopherol[28]. It is likely that the majority of γ-tocotrienol can be converted to α-tocotrienol by overexpression of both HGGT and γ-tocopherol methyltransferase in transgenic plants. The net result would be a considerable enhancement of dietary vitamin E activity, as has been shown in metabolic engineering studies with the tocopherol biosynthetic pathway[23,28].

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# Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content

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**Tocotrienols are the primary form of vitamin E in seeds of most monocot plants, including cereals such as rice and wheat. As potent antioxidants, tocotrienols contribute to the nutritive value of cereal grains in human and livestock diets. cDNAs encoding homogentisic acid geranylgeranyl transferase (HGGT), which catalyzes the committed step of tocotrienol biosynthesis, were isolated from barley, wheat and rice seeds. Transgenic expression of the barley HGGT in *Arabidopsis thaliana* leaves resulted in accumulation of tocotrienols, which were absent from leaves of nontransformed plants, and a 10- to 15-fold increase in total vitamin E antioxidants (tocotrienols plus tocopherols). Overexpression of the barley HGGT in corn seeds resulted in an increase in tocotrienol and tocopherol content of as much as six-fold. These results provide insight into the genetic basis for tocotrienol biosynthesis in plants and demonstrate the ability to enhance the antioxidant content of crops by introduction of an enzyme that redirects metabolic flux.**

The vitamin E family of antioxidants in plants consists of tocotrienols and tocopherols. Tocotrienols are the major form of vitamin E in seeds of most monocots[1] and a limited number of dicots[2]. Tocopherols, however, occur more widely than tocotrienols in plants and are the principal vitamin E components of leaves as well as seeds of most dicot species[1,3]. Tocotrienols typically account for >50% of the total vitamin E antioxidants of the seed endosperm of monocots, including palm and agronomically important cereal grains such as rice, wheat and oats[1,4,5].

Tocotrienols and tocopherols contain a polar chromanol ring linked to an isoprenoid-derived hydrocarbon chain (Fig. 1). The structure of tocotrienols differs from that of tocopherols by the presence of three *trans* double bonds in the hydrocarbon tail. In addition, α, β, γ and δ species of both tocopherols and tocotrienols differ with regard to the numbers and positions of methyl groups on the chromanol ring (Fig. 1).

Vitamin E is a generic term that refers to any of the eight naturally occurring forms of tocotrienols and tocopherols. These molecules can display a diversity of biological and physiological properties. Like tocopherols, tocotrienols are potent antioxidants[3,6,7] and are likely to protect plant cells against oxidative stresses such as those arising from the breakdown of polyunsaturated fatty acids in seed oils. The antioxidant capacity of tocotrienols also contributes to the nutritive value of food products and animal feeds derived from cereal grains[8,9]. In this regard, tocotrienols have been shown to have greater ability than tocopherols to scavenge free radicals and reduce lipid peroxidation in model membrane systems, but are not as readily absorbed by the body as α-tocopherol[6,7,10]. Tocotrienols have also been linked with a number of beneficial therapeutic properties, including the ability to reduce serum cholesterol levels[9,11–14] and to inhibit the growth of breast cancer cells[11,15,16]. Based on their reported health-promoting properties, tocotrienols are commercially produced as nutraceuticals from extracts of rice and palm oil.

The biosynthesis of tocotrienols has not been extensively studied in plants. Tocotrienols are generally believed to arise from the condensation of homogentisic acid (HGA) and geranylgeranyl diphosphate (GGDP), based on the structural similarity of the tocotrienol side chain and GGDP[17–19] (Fig. 1). The enzyme that catalyzes this reaction can therefore be designated as an HGA geranylgeranyl transferase (HGGT). In contrast to tocotrienols, the biosynthetic pathway of tocopherols has been well characterized in plants[17–20]. The committed step in tocopherol biosynthesis is the condensation of HGA and phytyl diphosphate (PDP), which is catalyzed by the plastid-localized enzyme HGA phytyltransferase (HPT)[17,18] (Fig. 1). cDNAs for this enzyme have recently been isolated from several dicot[19,20] and monocot species[19] as well as from the cyanobacterium *Synechocystis* (sp. PCC 6803)[21].

Notably, HPTs from dicots that accumulate only tocopherols do not display any detectable HGGT activity[18,22]. Extracts from chloroplasts of spinach leaves, for example, catalyze the condensation of HGA with PDP, but not with GGDP[18]. A similar strict substrate specificity for PDP has also recently been demonstrated for the *A. thaliana* HPT[19]. Results obtained from spinach chloroplasts led to the prediction that monocot seeds that accumulate tocotrienols contain a functionally divergent form of HPT (that is, HGGT; see Fig. 1) that displays substrate speci-

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